REMARKS

Applicants hereby amend Claim 21 for the purposes of clarification.

On pages 2-5 of the Office Action, Claims 2-8, 10-14, 21, 23-27 are rejected under 35 USC §103(a) as being obvious over Whitehead et al (U.S. Patent 4,554,088) in view of Ward et al, *FEMS Microbiology Letters* (1997).

Specifically, the Examiner contends that Whitehead et al teaches a simple binding assay that employs a ligand attached to a magnetic particle to capture the complementary ligand from a sample, wherein suitable ligand pairs include enzymes and their inhibitors. The Examiner further contends that it would have been obvious to use the generic assay system of Whitehead et al with an immobilized protein phosphatase enzyme in view of Ward et al, because Ward et al teaches that microcystins bind irreversibly to and inhibit protein phosphatases 1A and 2.

For the following reasons, Applicants respectfully traverse the Examiner's rejection.

Applicants respectfully submit that the disclosure of Whitehead et al has limitations, which weaken it as a starting point for this rejection. The techniques taught by Whitehead et al, while suitable for the use of immobilized antibodies in a binding assay, are not generally suitable for immobilized enzymes in an assay, such as the present invention. The reason for this is that there are fundamental differences between the properties of antibodies and those of enzymes. Antibodies are simple molecules which can be bound to a solid support by defined regions, and which will still retain their

function when immobilized. Enzymes, on the other hand, have complex conformational requirements in order to bind ligands and function correctly. Certain regions of enzymes may require proximity to other regions, which can only be achieved by a particular folded conformation. In addition, some regions of enzymes may require flexibility and/or particular steric interactions. Immobilizing an enzyme on a support in the manner of Whitehead et al could disrupt the required conformation of the enzyme, and therefore also its normal binding capabilities. The difficulties and unpredictability associated with immobilizing enzymes to perform the assay of Whitehead et al would have been known to one of ordinary skill in the art.

The discussion in Whitehead et al of the use of immobilized enzyme systems can be found in section 6.4 thereof. However, this is merely a general "prophetic" discussion. An example is found in section 7.13, which details the coupling of two particular enzymes to magnetic beads and the subsequent standard biochemical enzymatic assays that were performed. However these assays, in contrast to the radioimmunoassays exemplified in sections 7.5, 7.6, 7.7, 7.11 and 7.12 and the simple protein-cofactor binding assay exemplified in section 7.9, are not supported by any data. This leads one to speculate that the data obtained does not demonstrate a successful assay. A skilled reader would be skeptical about the lack of data for this enzymatic assay as compared to the immunoassays and would be put off considering the Whitehead et al method in an enzymatic context.

The lack of data proving that the method of Whitehead et al works for immobilized enzymes would result in the skilled man

having no motivation to combine the teachings of Whitehead et al and Ward et al. There would have been no reasonable expectation of success when considering the possibility of an assay in which a protein phosphatase is immobilized on a solid support, as claimed in the present application. Therefore, moving away from the solution method of Ward et al would not have been an obvious thing to do. Whitehead et al is only encouraging to the skilled reader for an antibody based method, not an enzyme based method.

The Examiner is requested to note that PP2A structure has only recently been determined (see Xing et al, *Cell*, 127:341-353 (2006); a copy of which is attached hereto). The Discussion section in this article focuses on the specific flexibility requirements of the PP2A conformation for its function.

The Examiner has previously maintained that the combination of the generic assay of Whitehead et al with the specific ligand pairs disclosed in Ward et al leads to the present invention. However, Carmichael (1994), which was published before Ward et al, discloses an enzymatic inhibition assay and also that microcystins bind to protein phosphatases. Carmichael et al discusses the disadvantages of the enzymatic assay, and suggests that a combination of ELISA and the enzymatic assay would prove very useful in detecting many of the toxins in an environmental sample. In other words, despite having the knowledge of Whitehead et al, Carmichael advises to use Whitehead et al's method and an enzymatic assay similar to Ward et al's "sequentially". When faced with the same problem, Ward et al also utilized an enzymatic assay and also acknowledges its limitations. The solution in Ward et al to the limitations of the enzymatic assay is to use the method "in

parallel" with other analytical techniques. Clearly then, the combination of Whitehead et al and Ward results at best in a "sequential" and separate use of the two methods.

The Examiner has acknowledged that a long-felt need exists, but he objects that other references address this need, citing Sikorska in particular. However, Sikorska discloses monoclonal antibodies used for measuring okadaic acid. The major disadvantage of immunoassays, that they do not accurately reflect the toxicity of a sample, has previously been explained to the Examiner, i.e., using antibodies in the manner disclosed in Sikorska will not directly detect toxicity. On the other hand, the method of the present invention does not have this drawback.

In any event, Applicants respectfully submit that the binding assay of the present invention provides unexpectedly superior results compared to both enzyme (ELISA) assays and protein phosphatase inhibition assays. Specifically, antibody assays, such as an ELISA, do not accurately reflect the toxicity of a sample, and enzymatic assays are prone to interference by contaminating factors.

Whitehead et al discloses only a simple binding assay for the detection of a ligand. On the other hand, the present invention relates to a competition assay for the detection of a ligand (the inhibitor/toxin) via the measurement of the second ligand. Thus, even if Whitehead et al was combined with Ward et al, the present invention would not be obtained. The use of immobilized protein phosphatase enzymes in a binding assay for the detection of toxins is not taught or suggest by

the combination of Whitehead et al and Ward et al, because Ward et al only discloses enzymatic inhibitions assays.

At paragraph 24 of the Office Action, the Examiner notes that Applicants' demonstration of a superior assay would be considered persuasive if it were not for the fact that Whitehead et al teaches the use of immobilized enzymes.

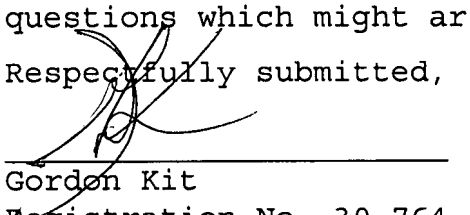
As discussed above, Applicants submit that the skilled reader would largely disregard the teaching of Whitehead et al as it relates to enzymes, and so there is no motivation to use the Whitehead et al method with the Ward et al phosphatase. Thus, the relevance of Applicants' advantages should not be ignored by the Examiner.

Accordingly, Applicants respectfully submit that the present invention is not taught or suggest in Whitehead et al, and that the combination thereof with Ward et al can only be made in hindsight, which is legally improper. Thus, Applicants request withdrawal of the Examiner's rejection.

In view of the amendments to the claims, and the arguments set forth above, reexamination, reconsideration and allowance are requested.

The Examiner is invited to contact the undersigned at his Washington telephone number on any questions which might arise.

Respectfully submitted,



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Structure of Protein Phosphatase 2A Core Enzyme Bound to Tumor-Inducing Toxins

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SUMMARY

The serine/threonine phosphatase protein phosphatase 2A (PP2A) plays an essential role in many aspects of cellular functions and has been shown to be an important tumor suppressor. The core enzyme of PP2A comprises a 65 kDa scaffolding subunit and a 36 kDa catalytic subunit. Here we report the crystal structures of the PP2A core enzyme bound to two of its inhibitors, the tumor-inducing agents okadaic acid and microcystin-LR, at 2.6 and 2.8 Å resolution, respectively. The catalytic subunit recognizes one end of the elongated scaffolding subunit by interacting with the conserved ridges of HEAT repeats 11–15. Formation of the core enzyme forces the scaffolding subunit to undergo pronounced structural rearrangement. The scaffolding subunit exhibits considerable conformational flexibility, which is proposed to play an essential role in PP2A function. These structures, together with biochemical analyses, reveal significant insights into PP2A function and serve as a framework for deciphering the diverse roles of PP2A in cellular physiology.

INTRODUCTION

Reversible protein phosphorylation is a fundamental regulatory mechanism in all aspects of biology (Hunter, 1995). In contrast to a large number of protein tyrosine phosphatases in the human genome (Alonso et al., 2004), there are only a few serine/threonine protein phosphatases, which are classified into three structurally distinct families. Protein phosphatase 2A (PP2A) belongs to the PPP family and is a major serine/threonine phosphatase involved in many essential aspects of cellular function (Janssens and Goris, 2001; Lechward et al., 2001; Virshup, 2000). PP2A plays an important role in cell-cycle regulation, cell growth control, development, regulation of multiple signal transduction pathways, cytoskeleton dynamics, and cell mobility. The catalytic subunit of PP2A is among the most conserved enzymes in species ranging from yeast to mammal (Cohen et al., 1990). PP2A is also an important tumor-suppressor protein (Janssens et al., 2005).

Despite its significance, mechanistic understanding of PP2A has been slow to emerge. This is at least in part due to the complex composition and regulation of PP2A. The PP2A core enzyme consists of a 36 kDa catalytic subunit, or C subunit, and a 65 kDa scaffolding protein, known as the A or PR65 subunit (Figure 1A). In mammalian cells, the A and C subunits each have two isoforms, α and β, which share very high sequence similarity (Arino et al., 1988; Green et al., 1987; Hemmings et al., 1990; Stone et al., 1987). In both cases, the α isoform is much more abundant than the β isoform. The PP2A core enzyme associates with a variable regulatory subunit to form a PP2A holoenzyme (Figure 1A). The variable regulatory subunits have four subfamilies: B (PR55), B′ (B56 or PR61), B″ (PR72), and B‴ (PR93/PR110), with at least 16 members in these subfamilies (Janssens and Goris, 2001; Lechward et al., 2001).

The PP2A core enzyme is a physiologically relevant and abundant entity in cells, rather than just an intermediate toward PP2A holoenzyme (Kremmer et al., 1997). Consequently, the PP2A core enzyme is regulated by numerous cellular proteins (Janssens and Goris, 2001). For example, the PP2A core enzyme interacts with a specific leucine carboxyl methyltransferase (LCMT), which methylates the carboxylate group of the carboxy-terminal residue Leu309 in the catalytic subunit of the PP2A core enzyme (Figure 1A). This methylation step plays an important role in allowing PP2A core enzyme to form a holoenzyme with the regulatory B subunit (Bryant et al., 1999; Kloeker et al., 1997; Tolstykh et al., 2000; Wei et al., 2001; Wu et al., 2000; Yu et al., 2001). On the other hand, the fully methylated PP2A core enzyme is the substrate for PP2A-specific methyl esterase (PME), which specifically removes the methyl group from Leu309 of the catalytic

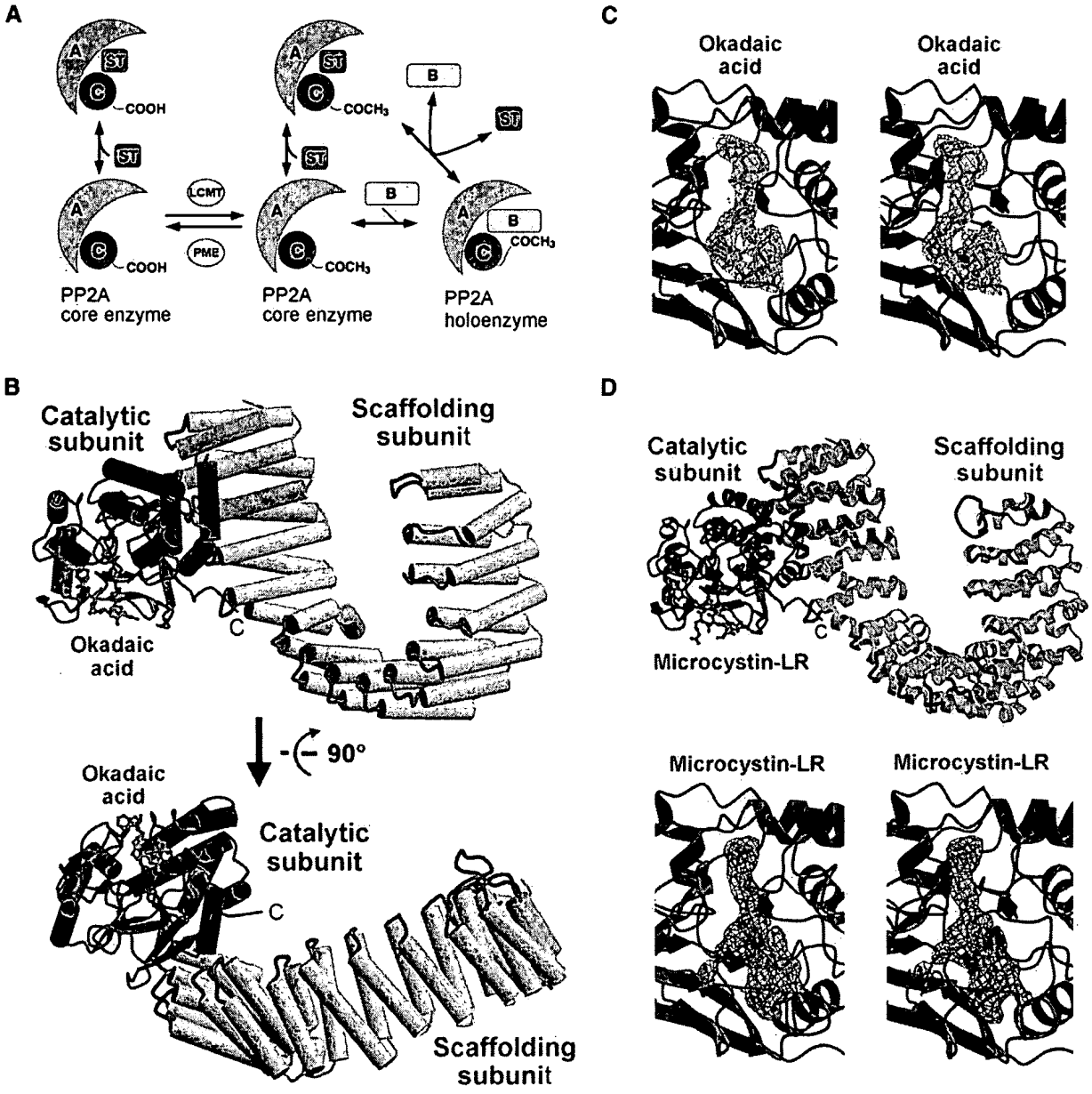

Figure 1. Overall Structure of the PP2A Core Enzyme Bound to Tumor-Inducing Toxins

(A) A schematic diagram describing the PP2A system. An essential component of the entire PP2A system is the PP2A core enzyme, which consists of a 65 kDa scaffolding subunit and a 36 kDa catalytic subunit. A, B, C, and ST denote the scaffolding subunit, regulatory subunit, catalytic subunit, and SV40 small tumor antigen, respectively.

(B) Overall structure of the PP2A core enzyme bound to okadaic acid (OA). The scaffolding subunit and catalytic subunit are shown in green and blue, respectively. OA is highlighted in yellow. The conserved interhelical region within each HEAT repeat is shown in magenta. Two perpendicular views are shown here.

(C) Stereoview of the bound OA. The $2F_o - F_c$ omit electron density, which was calculated after simulated annealing with the omission of OA, is contoured in yellow at 1.5σ around OA.

(D) Structure of the PP2A core enzyme bound to microcystin-LR (MCLR). The overall structure is shown in the top panel. The $2F_o - F_c$ omit electron density is contoured in magenta at 1.5σ around MCLR in the bottom panel. Figures 4A, 4B, and 4D were prepared using GRASP (Nicholls et al., 1991); all other structural figures were made using MOLSCRIPT (Kraulis, 1991).

subunit (Figure 1A). The phosphatase activity and specificity of the PP2A core enzyme are also subject to regulation by PP2A phosphatase activator (PTPA, also known as phosphotyrosyl phosphatase activator) (Chao et al., 2006; Janssens and Goris, 2001). The central importance of the PP2A core enzyme is further manifested by the

observation that SV40 and polyoma DNA tumor viruses transform cells in part by disrupting the function of the PP2A core enzyme (Mumby, 1995). The small tumor antigen of SV40 and the small and middle tumor antigens of polyoma virus compete with the regulatory B subunits for binding to the PP2A core enzyme (Figure 1A) (Joshi and Rundell, 1990; Pallas et al., 1990; Walter et al., 1990b).

PP2A is a tumor suppressor. Inactivation of both the α and β isoforms of the PP2A scaffolding subunit has been linked to cancer. Mutations in the scaffolding subunit that result in compromised binding to the regulatory or catalytic subunit of PP2A or a total absence or substantial reduction of the scaffolding subunit are closely associated with a variety of primary human tumors (Calin et al., 2000; Colella et al., 2001; Ruediger et al., 2001a; Suzuki and Takahashi, 2003; Takagi et al., 2000; Wang et al., 1998). In addition, an N-terminally truncated form of the B subunit PR61/B′γ1 was found to be associated with a higher metastatic state of melanoma cells (Ito et al., 2000, 2003; Koma et al., 2004), whereas gain- and loss-of-function experiments established this regulatory subunit as a tumor suppressor (Chen et al., 2004).

The concept that PP2A is a tumor suppressor was suggested by the discovery that the catalytic subunit of PP2A is the cellular target of okadaic acid (OA), a cocarcinogen and potent tumor inducer (Bialojan and Takai, 1988). OA exhibits an inhibitory constant (IC_{50}) of approximately 0.1 nM for PP2A and 10 nM for the related phosphatase PP1 (MacKintosh et al., 1990). Another toxin, the cyclic heptapeptide microcystin-LR (MCLR), also potently inhibits the activity of PP2A. Chronic exposure to MCLR through drinking water has been linked to liver cancer and high mortality rate in China (Harada et al., 1996; Ueno et al., 1996).

The PP2A scaffolding subunit contains 15 tandem repeats of a conserved 39-residue sequence known as a HEAT (huntingtin-elongation-A subunit-TOR) motif (Hemmings et al., 1990; Walter et al., 1990a). These 15 HEAT repeats are organized into an extended, horseshoe-shaped molecule (Groves et al., 1999). Previous studies showed that the conserved loop regions of HEAT repeats 11–15 are involved in binding to the catalytic subunit (Ruediger et al., 1992, 1994). To decipher the function and mechanism of PP2A, it is essential to elucidate the structure of the PP2A core enzyme, which has remained elusive despite intense investigation. In this manuscript, we report two crystal structures of the PP2A core enzyme, one bound to OA and the other to MCLR, and associated structural and biochemical analyses.

RESULTS

Assembly and Crystallization of PP2A Core Enzyme

The full-length α isoform of the PP2A catalytic subunit was overexpressed in baculovirus-infected insect cells and purified to homogeneity by affinity chromatography. The α isoform of the scaffolding subunit was expressed in bacteria as a fusion protein with glutathione S-transferase (GST) and immobilized on glutathione resin. The purified catalytic subunit was captured by the immobilized scaffolding subunit; the assembled PP2A core enzyme was released by thrombin cleavage and further purified on ion-exchange chromatography. Using phosphorylase a as the substrate, the recombinant PP2A core enzyme exhibits a level of catalytic activity identical to PP2A purified from bovine brain (data not shown). The phosphatase activity of the PP2A core enzyme was efficiently inhibited by a stoichiometric amount of OA or MCLR (data not shown).

The PP2A core enzyme was separately incubated with 1.2 molar equivalence of OA or MCLR for crystallization. After extensive trials, we succeeded in obtaining crystals of the 100 kDa PP2A core enzyme separately bound to OA and MCLR under similar conditions. The structures were determined by molecular replacement and refined to 2.6 and 2.8 Å resolution, respectively, for the OA- and MCLR-bound PP2A core enzyme (Table 1).

Overall Structure of the PP2A Core Enzyme

The structure of the PP2A core enzyme exhibits an extended architecture, measuring 130 Å in length, 80 Å in height, and 60 Å in width (Figure 1B). As previously shown (Groves et al., 1999), the scaffolding subunit forms an elongated, horseshoe-shaped structure characterized by double-layered α helices. There are 15 HEAT repeats in the scaffolding subunit, with each HEAT repeat comprising a pair of antiparallel α helices. The interhelical region within each HEAT repeat forms a contiguous ridge—hereafter referred to as the ridge—that is poised to bind other proteins. The catalytic subunit binds to one end of the scaffold through interactions with the ridge of HEAT repeats 11–15 (Figure 1B), consistent with an earlier prediction (Ruediger et al., 1992). Interactions between the scaffolding and the catalytic subunits result in the burial of 2072 $Å^2$ of otherwise exposed surface area.

The catalytic subunit adopts an α/β fold typical of the PPP family of phosphatases and contains two metal atoms at the active site (Figure 1B and Figure 2). Element analysis by inductively coupled plasma-emission spectrometry revealed the identity of the two metal atoms to be manganese. The active-site pocket of the catalytic subunit, occupied by OA (Figure 1C) or MCLR (Figure 1D), is positioned away from the ridge and is tilted toward the other end of the scaffolding subunit, where the regulatory B subunit and the substrate proteins presumably bind. The carboxyl terminus of the catalytic subunit is also pointing toward the other end of the scaffolding subunit, consistent with its reported role in recruiting the regulatory B subunit. The binding sites for OA (Figure 1C) and MCLR (Figure 1D) are completely overlapping; a similar set of amino acids in the catalytic subunit are involved in interactions with both toxins (Figure 2).

Structures of a number of related protein serine/threonine phosphatases such as those of PP1 and PP5 have been determined (Egloff et al., 1995; Goldberg et al., 1995; Kita et al., 2002; Maynes et al., 2001; Swingle et al., 2004; Terrak et al., 2004; Yang et al., 2005). Although

Table 1. Statistics from Crystallographic Analysis

	PP2A + OA	PP2A + MCLR
Beamline	NSLS-X25	NSLS-X25
Space group	I222	I222
Resolution (Å)	50.0–2.5 Å	50.0–2.8 Å
Total observations	299,020	238,497
Unique observations	62,598	44,149
Data coverage (outer shell)	99.4% (100.0%)	98.3% (97.0%)
R_{sym} (outer shell)	0.070 (0.465)	0.061 (0.400)
Refinement		
Resolution range (Å)	30.0–2.6 Å	30.0–2.8 Å
Number of reflections ($\|F\| > 0$)	53,380	42,363
Data coverage	94.9%	92.9%
R_{work}/R_{free}	0.226/0.270	0.222/0.264
Total number of atoms	6,988	6,956
Number of waters	64	22
Rmsd bond length (Å)	0.010	0.010
Rmsd bond angles (°)	1.60	1.53
Ramachandran Plot		
Most favored (%)	87.5	86.3
Additionally allowed (%)	11.2	12.2
Generously allowed (%)	1.1	1.3
Disallowed (%)	0.2	0.2

$R_{sym} = \Sigma_h\Sigma_i|I_{h,i} - I_h|/\Sigma_h\Sigma_i I_{h,i}$, where I_h is the mean intensity of the i observations of symmetry-related reflections of h. $R = \sum|F_{obs} - F_{calc}|/\sum F_{obs}$, where $F_{obs} = F_P$ and F_{calc} is the calculated protein structure factor from the atomic model. R_{free} was calculated with 5% of the reflections. Rmsd bond lengths and angles are the deviations from ideal values.

the overall structure of the catalytic subunit of PP2A resembles those of these related phosphatases, significant local structural differences remain, which are primarily distributed in solvent-exposed surface loops (Figure 2B). These differences result in a relatively large root-mean-square-deviation (rmsd) of 3.38 Å over all 284 aligned Cα atoms between PP2A and PP1. These local structural differences determine the functional diversity between PP2A and other related phosphatases.

Interface between the Catalytic and Scaffolding Subunits

The extended architecture of the PP2A core enzyme contrasts with strong and specific interactions between the catalytic subunit and the scaffolding subunit. Despite significant sequence similarity between the catalytic subunit of PP2A and other serine/threonine phosphatases (Figure 2), the scaffolding subunit of PP2A only recognizes the catalytic subunit of PP2A. To understand the underlying molecular basis for the specific assembly of the PP2A core enzyme, we analyzed the interface between the catalytic subunit and the scaffolding subunit of PP2A (Figure 3). The interface primarily involves HEAT repeats 11–15 in the scaffolding subunit and the region surrounding helix α2 and the carboxy-terminal fragment of the catalytic subunit (Figure 2). The recognition specificity is provided by 15 intermolecular hydrogen bonds and significant van der Waals contacts (Figures 3B and 3C).

The linear arrangement of HEAT repeats allows the entire interface to be divided into two consecutive segments for easy description. One segment involves HEAT repeats 11 and 12. At the center of this portion of the interface, Trp417 from HEAT11 and Leu455 from HEAT12 make multiple van der Waals contacts to Arg70 and Ile71 on helix α2 of the catalytic subunit (Figure 3B). At the periphery, four hydrogen bonds, one of which occurs between the side chains of Arg418 and Glu67, further strength the interaction (Figure 3B). Interestingly, Arg418 was mutated to Trp in a melanoma-derived cDNA, which was shown to have a negative impact on interaction with the catalytic subunit (Ruediger et al., 2001b). The other segment involves HEAT repeats 13, 14, and 15. Although van der Waals interactions also play a significant role, hydrogen bonds dominate at this portion of the interface (Figure 3C). Inter- and intramolecular hydrogen bonds interdigitate to form two extensive networks. In one network, the carboxylate side chain of Asp280 of the catalytic subunit makes four intermolecular hydrogen bonds: one to the carbonyl oxygen atom of residue 493, one to the backbone amide of residue 495, and two to the side chain of Arg498 in the scaffolding subunit (Figure 3C). These interactions are buttressed by four intramolecular hydrogen bonds to the side chain of Arg498: a pair of charge-stabilized contacts from the carboxylate side chain of Asp531 and two additional contacts from the carbonyl groups of residues 492 and 493 (Figure 3C). In the other network, the side chain of Asn535 of the scaffolding subunit makes four intermolecular hydrogen bonds: two to the side chain of Asn79 and two to the carbonyl oxygen atoms of residues 76 and 77 of the catalytic subunit (Figure 3C). Val533 of the scaffolding subunit, the equivalent of which in the β isoform (Val545) is mutated to Ala in colon cancer (Wang et al., 1998), resides at the center of this portion of the interface and interacts with Pro51 in the catalytic subunit. Consistent with the structural observation, V545A in the β isoform of the scaffolding subunit resulted in decreased binding to the regulatory B″/PR72 subunit (Ruediger et al., 2001a).

A number of interface residues in the catalytic subunit of PP2A that play an important role in binding to the scaffolding subunit are substituted by nonconserved residues in other related phosphatases. For example, the inability of PP1, PP2B, PP5, and PP7 to bind to the PP2A scaffolding

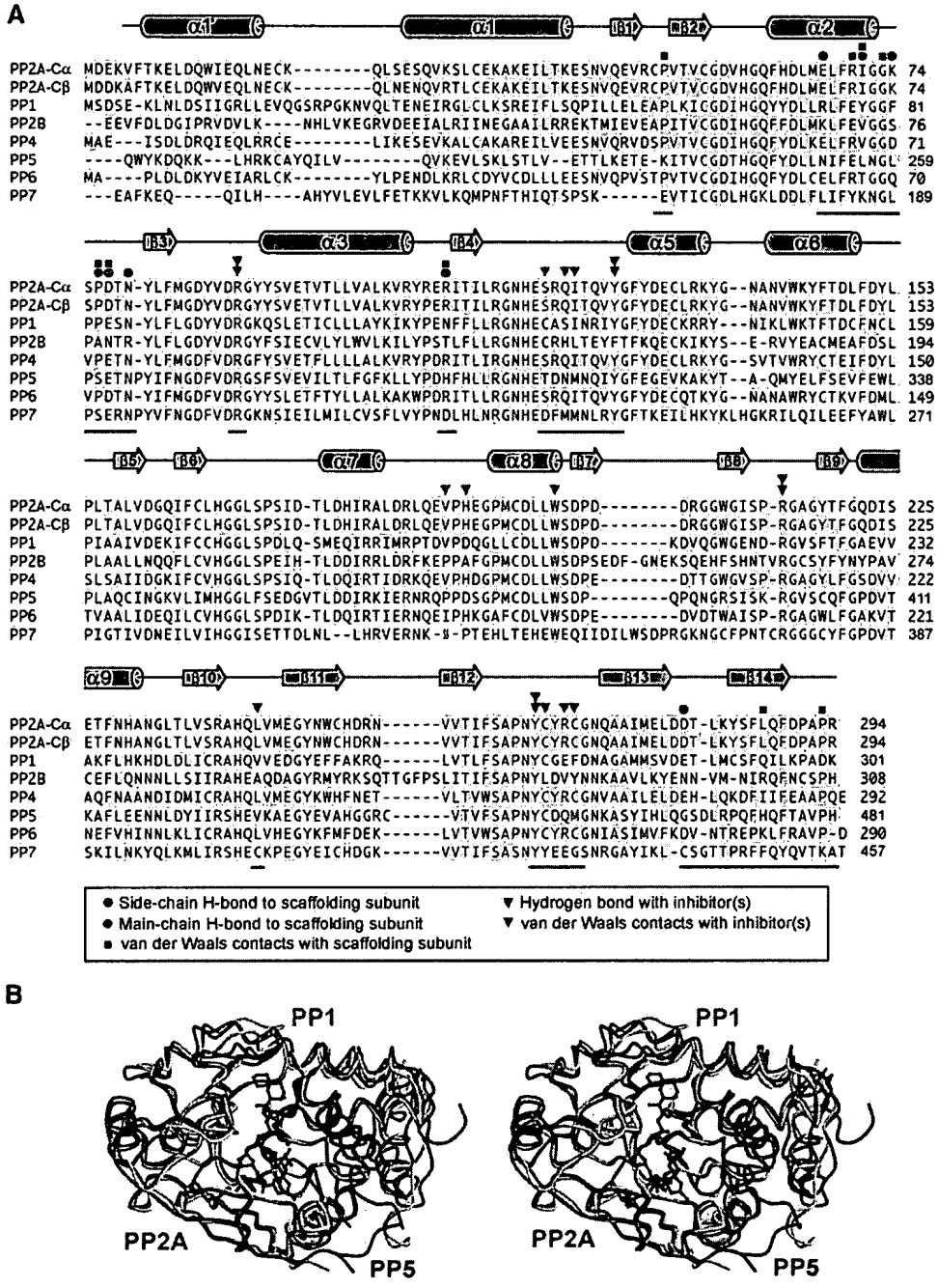

Figure 2. Conservation and Diversity of Sequences and Structures

(A) Sequence alignment of the catalytic subunit of PP2A with other homologous phosphatases of human origin. Both α and β isoforms of PP2A are shown. Secondary structural elements of the catalytic subunit of PP2A are indicated above the sequences. Conserved residues are highlighted in yellow. Residues that make hydrogen bonds to the scaffolding subunit via their side-chain and main-chain atoms are identified by red and green circles, respectively. Residues that contribute to van der Waals interactions with the scaffolding subunit are denoted by blue squares. Residues that specifically recognize the toxins through hydrogen bonds and van der Waals contacts are shown in red and yellow triangles, respectively.

(B) Overlay of structure of the PP2A catalytic subunit with those of PP1 and PP5. The structures of PP2A, PP1 (PDB ID code 1FJM), and PP5 (PDB ID code 1S95) are shown in blue, yellow, and magenta, respectively. OA (bound to PP2A) is shown in black.

subunit appears to be a consequence of sequence variation at positions corresponding to Glu67, Arg70, Arg110, and Asp280 in the PP2A catalytic subunit (Figure 2). While these residues are conserved in PP4 and PP6, these phosphatases diverge at Lys74, which donates a hydrogen bond to Tyr456 in HEAT repeat 12 of the PP2A

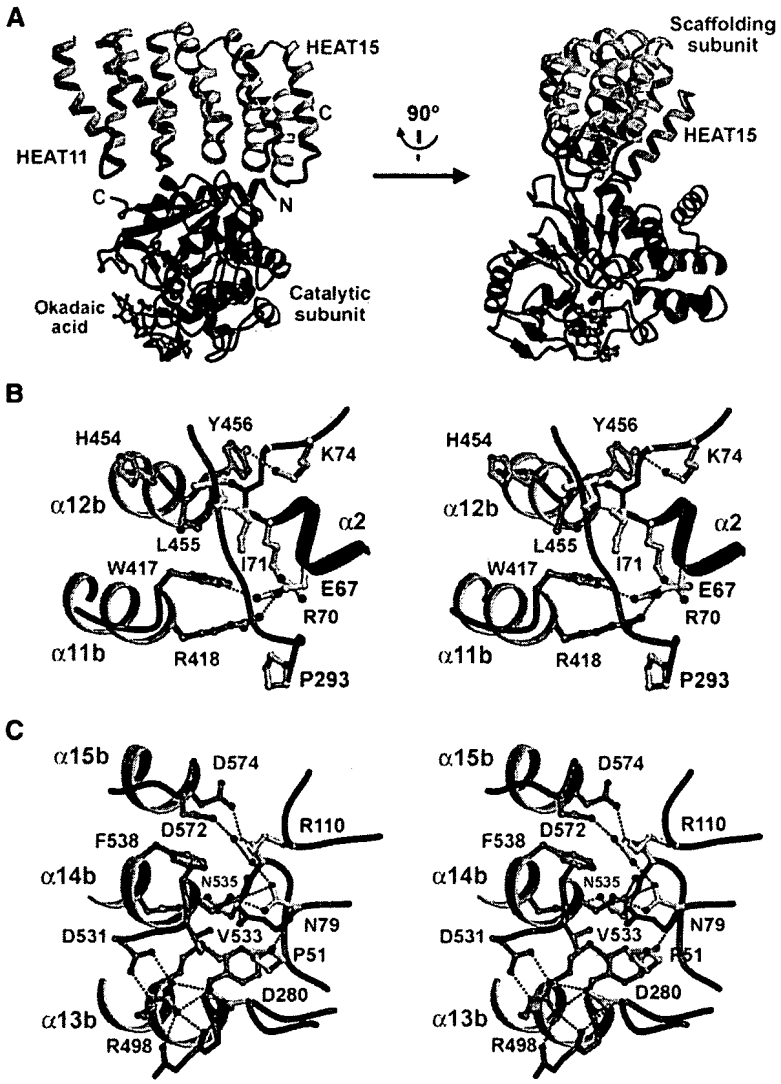

Figure 3. Specific Interactions between the Scaffolding Subunit and the Catalytic Subunit

(A) The catalytic subunit of PP2A only interacts with HEAT repeats 11–15 of the scaffolding subunit. Two perpendicular views of the interface are shown. The color scheme is the same as in Figure 1B.

(B) Stereoview of the atomic interactions between the catalytic subunit and HEAT repeats 11 and 12 of the scaffolding subunit. Side chains from the catalytic subunit and scaffolding subunit are colored yellow and orange, respectively. Hydrogen bonds are represented by red dotted lines.

(C) Stereoview of the atomic interactions between the catalytic subunit and HEAT repeats 13–15 of the scaffolding subunit. This interface is dominated by two extensive networks of hydrogen bonds.

scaffolding subunit (Figure 2). This analysis provides a molecular explanation for the specificity of the scaffolding subunit toward the catalytic subunit of PP2A.

Toxin Binding

Despite their different chemical identity, both OA and MCLR bind to the same surface pocket on the catalytic subunit of PP2A (Figures 4A and 4B). This observation is consistent with the report that preincubation of PP2A with OA prevented subsequent binding to MCLR (MacKintosh et al., 1990). The binding pocket is located right above the two manganese atoms and the active site of PP2A. As previously noted for PP1 (Goldberg et al., 1995; Moorhead et al., 1994), interactions between PP2A and MCLR are strengthened by a covalent linkage between the Sγ atom of Cys269 and the terminal carbon atom of the Mdha side chain (Figure 4B).

Remarkably, an identical set of amino acids in the catalytic subunit of PP2A mediate a similar set of interactions with both inhibitors. For example, the guanidium group of Arg89 donates two hydrogen bonds to different oxygen atoms in OA (Figure 4A) and in MCLR (Figure 4B). Similarly, Tyr265 also contributes hydrogen bonds in both toxin-bound complexes. On one end of the binding pocket, four amino acids in the catalytic subunit of PP2A, Gln122, Ile123, His191, and Trp200, form a hydrophobic cage, which accommodates the long hydrophobic Adda side chain in MCLR (Figure 4B) and the hydrophobic end of OA (Figure 4A). On the other end of the binding pocket, Leu243, Tyr265, Cys266, Arg268, and Cys269 make multiple van der Waals interactions with the hydrophobic portion of the bound toxins (Figures 4A and 4B).

The catalytic subunit of PP2A is the primary cellular target of OA (Bialojan and Takai, 1988). Okadaic acid exhibits an IC_{50} of approximately 0.1 nM for PP2A and 10 nM for PP1 (MacKintosh et al., 1990); PP1 and PP2A share approximately 50 percent sequence identity (Figure 2A). Comparison of our structure with that of PP1 bound to OA (Maynes et al., 2001) allowed rationalization of its strong preference for PP2A. Although many residues of

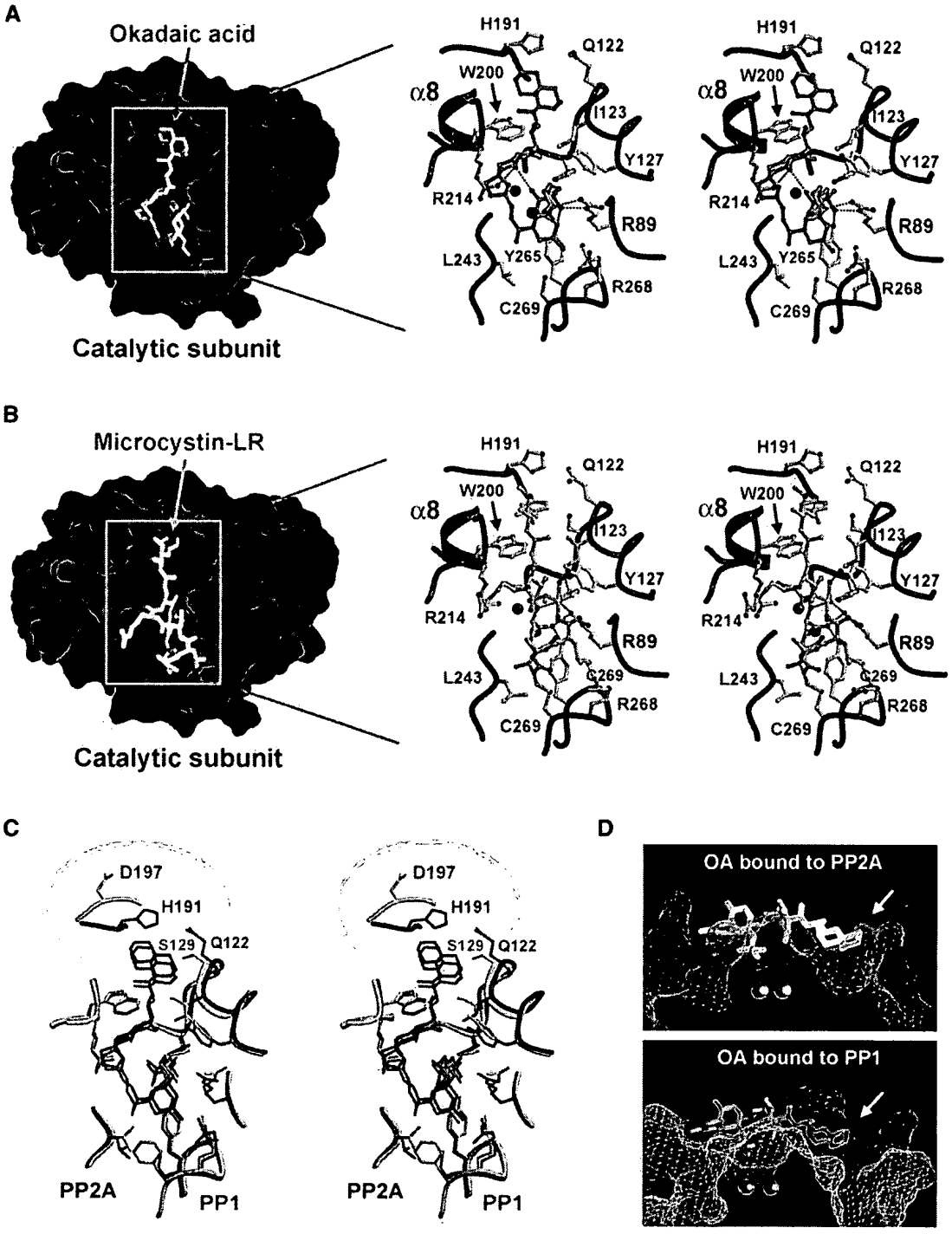

Figure 4. Specific Recognition of the Toxins by PP2A

(A) Specific recognition of OA. OA (in yellow) binds to the active-site pocket of the catalytic subunit (shown in blue surface representation) in the left panel. The two manganese atoms are shown as red spheres. The detailed atomic interactions are depicted in the right panel. Hydrogen bonds are represented by red dotted lines.

(B) Specific recognition of MCLR. MCLR (in green) binds to the same surface location as OA (left panel). The detailed atomic interactions are depicted in the right panel.

(C) Structural comparison of OA binding to PP2A (blue) and PP1 (yellow). This comparison reveals that the hydrophobic cage in the catalytic subunit of PP2A that accommodates the hydrophobic end of OA is absent in PP1, which may account for PP1's differential specificity for OA.

(D) A surface representation of OA binding to PP2A and PP1. A slice of PP1 or the catalytic subunit of PP2A is shown as transparent mesh. The two active-site manganese atoms are shown as red spheres. This comparison highlights the missing hydrophobic cage in PP1.

PP2A that specifically recognize OA are conserved in PP1 (Figure 2A), the hydrophobic cage in the catalytic subunit of PP2A that accommodates the hydrophobic end of OA is absent in PP1 (Figure 4C). His191, which resides on the intervening loop between helices α7 and α8 of PP2A, contributes to one side of the cage. Compared to His191, the corresponding residue Asp197 along with the corresponding loop in PP1 is located 4–5 Å further away from OA (Figure 4C). In addition, Gln122 of PP2A, whose aliphatic side chain contributes to another side of the hydrophobic cage, is replaced by Ser129 in PP1, leading to much diminished capacity in mediating van der Waals interaction (Figure 4C). The net effect of these substitutions is that PP1 contains an open-ended groove, whereas PP2A has a hydrophobic cage that better accommodates the hydrophobic end of OA (Figure 4D).

Conformational Flexibility of the Scaffolding Subunit

The conformation of the free scaffolding subunit is well defined, with only minor variation in the carboxy-terminal HEAT repeats (Groves et al., 1999). To investigate whether assembly of the PP2A core enzyme results in any significant alteration of the scaffolding subunit, we compared the conformation of the free scaffolding subunit with that in the PP2A core enzyme. Whereas HEAT repeats 1–12 from the two structures can be superimposed with an rmsd of 1.25 Å over 444 Cα atoms, HEAT repeats 13–15 are separated by as much as 20–30 Å (Figure 5A). In isolation, however, HEAT repeats 13–15 from the two structures can also be superimposed with an rmsd of 0.49 Å over 114 Cα atoms (Figure 5B). This analysis indicates that formation of the PP2A core enzyme results in a major conformational switch in the scaffolding subunit, which primarily occurs at a hinge region between HEAT repeats 12 and 13. This conclusion is consistent with the structural analysis of the interface between the catalytic subunit and the scaffolding subunit, which shows that the globular catalytic subunit makes extensive interactions with HEAT repeats 11–15 (Figure 3).

To accurately locate the site of the conformational change, we made a pairwise comparison of the 15 HEAT repeats between the free and the bound scaffolding subunits. HEAT repeats 2–10, 14, and 15 exhibit relatively small rmsds of 0.49 Å, 0.34 Å, 0.36 Å, 0.27 Å, 0.40 Å, 0.35 Å, 0.29 Å, 0.29 Å, 0.29 Å, 0.24 Å, and 0.23 Å, respectively. HEAT repeats 11 and 13 have moderate rmsds of 0.64 Å and 0.63 Å, respectively. In contrast, HEAT repeat 12 displays the largest conformational change, with an rmsd of 1.35 Å. Next, we examined the relative conformational change from one HEAT repeat to the next (Figure 5C). In this analysis, HEAT repeat N (shown as thin lines in Figure 5C) is superimposed between the free and the bound scaffolding subunits, and conformation of HEAT repeat N+1 is compared (shown as cylinders). This analysis revealed that significant conformational changes mainly occur at two HEAT interfaces: between repeats 11 and 12 and between repeats 12 and 13 (Figure 5C).

The large extent of conformational differences in the free and the bound scaffolding subunits is more appropriately described as structural changes or refolding of the scaffolding subunit. Compared to the free scaffolding subunit, packing interactions in the hydrophobic core between adjacent HEAT repeats have been significantly altered by the binding of the catalytic subunit. For example, Leu451 interacts with Phe503 and Cys504 at the center of the interface between HEAT repeats 12 and 13 in the free scaffolding subunit (Figure 5D, left panels). However, Leu451 interacts with Val486 and Cys504 instead in the scaffolding subunit of the PP2A core enzyme (Figure 5D, right panels). Val452, which makes multiple van der Waals contacts at the interface in the free scaffolding subunit (Figure 5D, left panels), is rotated completely out of the interface in the PP2A core enzyme (Figure 5D, right panels). Similarly, the hydrophobic core between HEAT repeats 11 and 12 has also been altered, albeit to a smaller extent compared to repeats 12 and 13. Thus, the scaffolding subunit of PP2A exhibits remarkable plasticity in the hydrophobic core between adjacent HEAT repeats.

The observed conformational flexibility in the scaffolding subunit of the PP2A core enzyme may have significant functional implications. Although the conformational flexibility mainly occurs in the HEAT repeats where the catalytic subunit binds, it is entirely possible that such flexibility is also inducible in other HEAT repeats by other regulatory components. We speculate that the conformational flexibility of the extended scaffolding subunit might be important for its interaction with other regulatory proteins and for the catalytic activity of the PP2A holoenzyme.

Assembly and Regulation of PP2A Holoenzyme

The PP2A core enzyme interacts with a regulatory B subunit to form a heterotrimeric PP2A holoenzyme (Figure 1A). The regulatory B subunits are classified into three major subfamilies: B (PR55), B′ (B56 or PR61), and B″ (PR72), each with at least four different isoforms (Janssens and Goris, 2001; Lechward et al., 2001). Unlike the scaffolding and catalytic subunits, sequence similarity between different subfamilies of the regulatory subunits is very low, and the expression levels of various B subunits are highly diverse depending upon cell types and tissues (Janssens and Goris, 2001; Lechward et al., 2001). In this regard, the B subunits determine the substrate specificity as well as the spatial and temporal functions of PP2A.

Methylation of the carboxy-terminal leucine residue in the catalytic subunit is thought to be important for the PP2A core enzyme to interact with the B/PR55 and B′/PR61 subunits (Bryant et al., 1999; Kloeker et al., 1997; Tolstykh et al., 2000; Wei et al., 2001; Wu et al., 2000; Yu et al., 2001). To examine whether this requirement also applies to the B″/PR72 subunits, we cloned, expressed, and purified the human α isoform of the B″/PR72 subfamily. Then we examined formation of a PP2A holoenzyme involving the B″/PR72-α subunit. The PP2A core enzyme was methylated by LCMT in the presence of S-adenosyl methionine (SAM). The extent of

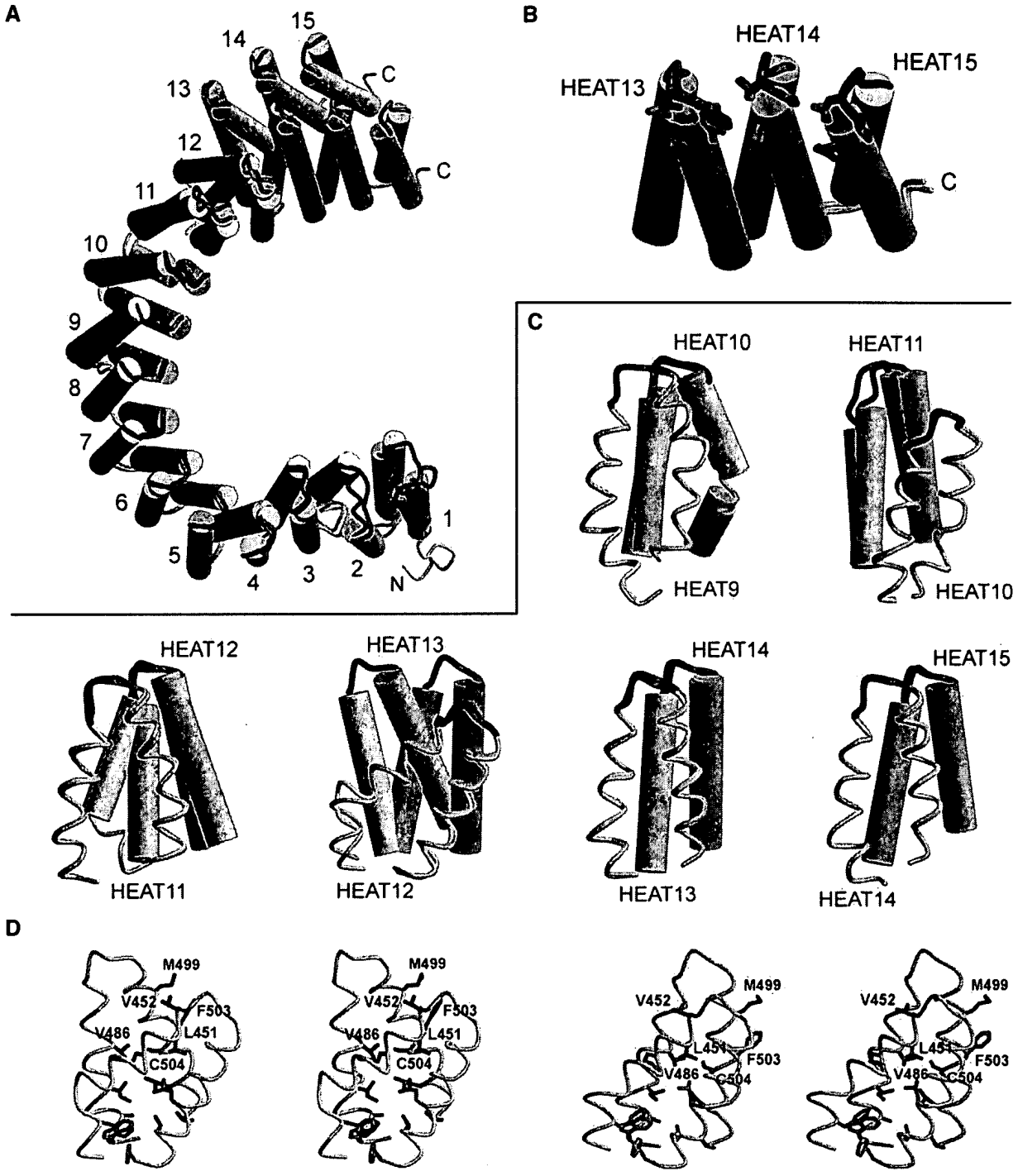

Figure 5. Conformational Flexibility of the Scaffolding Subunit

(A) Structural comparison of the free scaffolding subunit (yellow) and the scaffolding subunit from the PP2A core enzyme (green). The conserved interhelical region within each HEAT repeat is colored blue and magenta for the free scaffolding subunit and the bound scaffolding subunit, respectively. When the first 12 HEAT repeats are aligned, there is a large structural deviation in HEAT repeats 13–15.

(B) HEAT repeats 13–15 are aligned well between the free scaffolding subunit and the bound scaffolding subunit.

(C) Conformational changes from one HEAT repeat to the next. The HEAT repeats in the front (thin line) are aligned between the free scaffolding subunit and the bound scaffolding subunit, and the next HEAT repeats in the back (cylinder) are compared. This analysis reveals that the largest interrepeat conformational changes occur in HEAT repeats 12 and 13.

(D) Alteration of the hydrophobic core between HEAT repeats 12 and 13. Compared to the free scaffolding subunit (left stereoview), the packing interactions in the hydrophobic core between HEAT repeats 12 and 13 have been altered in the PP2A core enzyme (right stereoview).

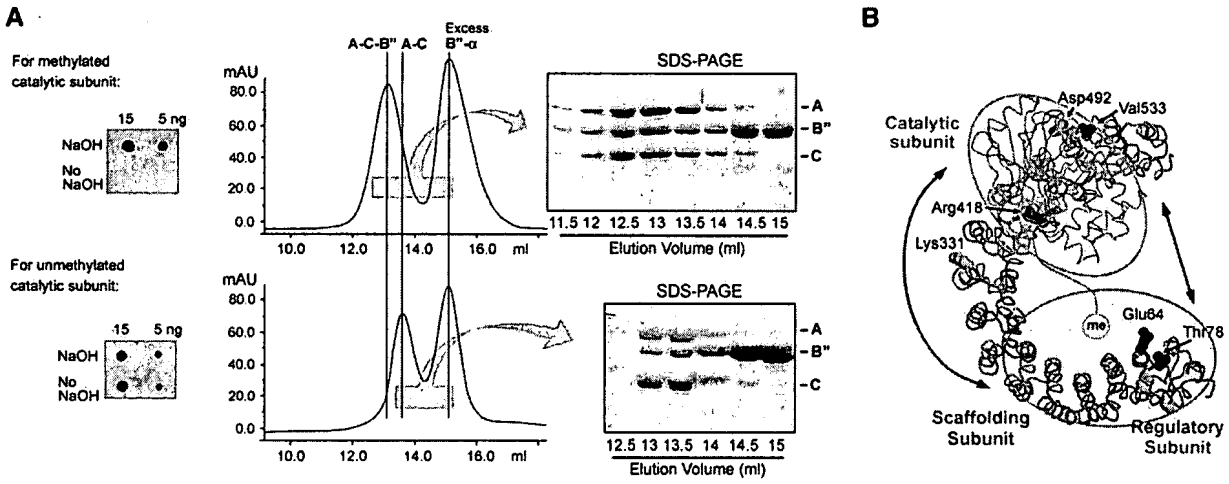

Figure 6. Assembly and Regulation of the PP2A Holoenzyme

(A) The B″/PR72 regulatory subunit only forms a stable complex with the methylated PP2A core enzyme. The left panels show results of a western blot for the PP2A core enzyme, using an antibody that specifically recognizes the unmethylated carboxyl terminus of the PP2A catalytic subunit. NaOH removes the methyl group. The right panels show results of gel filtration chromatography. The B″-α protein comigrates only with the methylated PP2A core enzyme.

(B) A model for the formation of PP2A holoenzyme. The regulatory subunit binds to the amino-terminal ten HEAT repeats of the scaffolding protein. The methylated carboxyl terminus of the catalytic subunit is predicted to make direct interactions to the regulatory subunit. Conformational flexibility of the scaffolding subunit (indicated by red arrows) is proposed to be essential for the function of PP2A. Amino acids predicted to be at the interface between the scaffolding subunit and other subunits are highlighted in red.

methylation was examined using an antibody that only specifically recognizes the unmethylated carboxyl terminus of the catalytic subunit of PP2A (Figure 6A, top left panel). Following complete methylation, the fully methylated PP2A core enzyme was incubated with an excess amount of the B″/PR72 protein; the mixture was analyzed by gel filtration chromatography. The methylated PP2A core enzyme coeluted with the B″/PR72 protein in a single peak, which corresponds to an apparent molecular mass of approximately 160 kDa (Figure 6A, top right panel). Importantly, the elution volume for this peak is smaller than that of the PP2A core enzyme or the B″/PR72 protein alone. This result unambiguously demonstrates that the methylated PP2A core enzyme forms a stable holoenzyme with the B″/PR72 protein. In contrast, the unmethylated PP2A core enzyme failed to form a stable complex with the B″/PR72 protein, as the PP2A core enzyme eluted from gel filtration at a volume identical to that of the free PP2A core enzyme (Figure 6A, bottom panels).

This result, together with our structural analysis and previous knowledge, gives rise to a model of the assembly and regulation of PP2A holoenzyme. In this model, the regulatory B subunit is expected to interact with the amino-terminal ten HEAT repeats of the scaffolding subunit (Figure 6B). The methylated carboxyl terminus of the PP2A catalytic subunit is predicted to make direct interactions with the regulatory B subunit. Compared to the free scaffolding subunit, binding by the catalytic subunit induces remarkable conformational changes in the carboxy-terminal HEAT repeats. Such changes bring the carboxyl terminus of the scaffolding subunit closer to its amino terminus. Likewise, binding by the regulatory subunit is predicted to induce additional conformational changes in the amino-terminal HEAT repeats. Such conformational flexibility (indicated by red arrows) is proposed to be important not only for binding to the catalytic and regulatory subunits but also for the catalytic activity of PP2A.

DISCUSSION

PP2A is one of the most important protein serine/threonine phosphatases. The PP2A core enzyme, consisting of the scaffolding subunit and the catalytic subunit, is at the center of PP2A regulation (Figure 1A). Importantly, the PP2A core enzyme is a physiologically relevant and abundant entity in cells rather than just an intermediate toward PP2A holoenzyme (Kremmer et al., 1997). Using several monoclonal antibodies against different regions of the scaffolding subunit, the PP2A core enzyme was shown to represent at least one-third of the total cellular PP2A protein (Kremmer et al., 1997). Compared to the PP2A holoenzyme, the PP2A core enzyme is differentially regulated by many interacting proteins. For example, the small tumor antigen of SV40 was shown to only interact with the PP2A core enzyme but not the holoenzyme (Yang et al., 1991). Consequently, the phosphatase activity of the PP2A core enzyme, but not the holoenzyme, was inhibited by the small tumor antigen (Scheidtmann et al., 1991). In addition, varying the ratio of PP2A core enzyme to holoenzyme was shown to have significant biological consequence (Ruediger et al., 1997).

In this manuscript, we report two crystal structures of the PP2A core enzyme bound to tumor-inducing toxins: one to OA and the other to MCLR, at 2.6 and 2.8 Å resolution, respectively. Our structure reveals that the catalytic subunit recognizes one end of the elongated scaffolding subunit by extensively interacting with the conserved ridges of HEAT repeats 11–15. The structural observation agrees nicely with previous mutagenesis studies that mapped the binding element for the catalytic subunit (Ruediger et al., 1994; Ruediger et al., 1992). The interface consists of networks of intermolecular hydrogen bonds as well as significant van der Waals contacts. At least two tumor-derived missense mutations, R418W in the α isoform and V545A in the β isoform, map to the interface. The important interactions mediated by Arg418 and Val533 (corresponding to Val545 in the β isoform) provide a satisfying explanation to the biochemical finding that these mutations crippled binding to the catalytic subunit (Ruediger et al., 2001a, 2001b). Because disruption of binding to the catalytic subunit prevents normal function of PP2A, other amino acids that play an important role at the interface are likely to be targeted by cancer-derived mutations. In this regard, structure of the PP2A core enzyme will serve as an important reference for understanding other yet to be uncovered cancer-derived mutations.

Our structures reveal the molecular basis for PP2A-mediated specific recognition of OA and MCLR, two potent tumor inducers. Both OA and MCLR occupy the same surface pocket and make extensive interactions with a similar set of amino acids lining the active site of the catalytic subunit. Structural comparison of OA binding by PP2A and by PP1 revealed that a hydrophobic cage in the catalytic subunit of PP2A that accommodates the hydrophobic end of OA is absent in PP1, providing a plausible explanation for the observed differences in inhibitory constants.

An important finding of this study is the remarkable conformational flexibility in the scaffolding subunit. Compared to the free scaffolding subunit, HEAT repeats 13–15 in the PP2A core enzyme are reoriented by as much as 20–30 Å. The conformational flexibility is most drastic between HEAT repeats 12 and 13, followed by that between repeats 11 and 12. The degree of conformational changes amounts to refolding of the hydrophobic core both between and within the HEAT repeats. These conformational changes appear to be brought about by the interactions between the scaffolding subunit and the catalytic subunit because the most drastic conformational shift (between HEAT repeats 12 and 13) is the center of the interface between the scaffolding subunit and the catalytic subunit. In addition to HEAT repeats 11–13, a small degree of conformational variation is also present both within and between other HEAT repeats. The observed conformational flexibility, an intrinsic property of the scaffolding subunit, is proposed to be essential to the function of PP2A in several aspects. First, conformational flexibility of the scaffolding subunit is required for binding to the catalytic subunit and possibly other interacting proteins such as the regulatory subunits. Second, conformational flexibility of the scaffolding subunit might be important for the phosphatase activity of the catalytic subunit. Dephosphorylation of cellular target proteins may require certain degrees of motion for the scaffolding subunit. The elongated shape and the relatively loose interrepeat packing are both ideally suited to meet such a requirement. We further speculate that one important role of the regulatory subunit is to position the bound substrate in close proximity to the catalytic subunit. Then, the conformational flexibility of the scaffolding subunit facilitates the dephosphorylation activity of the catalytic subunit.

Our structural analysis of the PP2A core enzyme greatly facilitates studies on the assembly and regulation of the PP2A holoenzyme. Previous studies have unequivocally demonstrated that methylation of the PP2A core enzyme is important for the formation of a holoenzyme involving the B/PR55 or B′/PR61 subunit (Bryant et al., 1999; Kloeker et al., 1997; Tolstykh et al., 2000; Wei et al., 2001; Wu et al., 2000; Yu et al., 2001). In this study, we provide strong evidence that formation of a stable holoenzyme involving the B″/PR72 subunit also requires methylation of the PP2A core enzyme (Figure 6A).

EXPERIMENTAL PROCEDURES

Protein Preparation and Assembly of PP2A Core Enzyme

All constructs and point mutations were generated using a standard PCR-based cloning strategy. The full-length PP2A Aα subunit (1–589), all missense mutants, and the regulatory B″/PR72 subunit were cloned into pGEX-2T vector (GE Healthcare) and overexpressed in *E. coli* strain BL21(DE3). The soluble fraction of the *E. coli* cell lysate was purified by glutathione resin (QIAGEN) and further fractionated by ion exchange (Source 15Q, Amersham). The full-length PP2A Cα subunit (1–309) was cloned into baculovirus transfer vector pVL1392 (Pharmingen) as an N-terminal 8×His-tagged protein. Recombinant baculovirus was generated using the BaculoGold cotransfection kit (Pharmingen). Cα was overexpressed in baculovirus-infected Hi-5 suspension culture, purified by Ni-NTA resin (QIAGEN) to homogeneity, and further fractionated by ion exchange (Source 15Q, Amersham).

The PP2A core enzyme was assembled by passing Cα, which was preincubated with an excess amount of MCLR or OA, through a stoichiometric amount of GST-Aα immobilized on glutathione resin. The A-C dimer was released by on-column thrombin cleavage and further purified by ion-exchange chromatography. Phosphatase assays were performed to ensure that there was no remaining activity for the PP2A core enzyme bound to inhibitor.

Crystallization and Data Collection

Crystals of the PP2A core enzyme were grown by the hanging-drop vapor diffusion method by mixing the protein complex (~10 mg/ml) with an equal volume of reservoir solution containing 0.2 mM lithium sulfate, 1.5 M ammonium sulfate, 0.1 M Tris-Cl (pH 8.5). Crystals appeared overnight and grew to full size within 3 days. The crystals belonged to space group I222, with a = 92.540 Å, b = 194.850 Å, c = 201.350 Å and one complex per asymmetric unit. Crystals were equilibrated in a cryoprotectant buffer containing reservoir buffer plus 20% glycerol (v/v) and were flash frozen in a cold nitrogen stream at −170°C. The native set was collected at NSLS beamline X25 and processed using the software Denzo and Scalepack (Otwinowski and Minor, 1997).

Structure Determination

The structure of human PP2A core enzyme was determined by molecular replacement. First, Cα was located using the program PHASER

(McCoy et al., 2005) and the atomic coordinates of a homologous phosphatase, PP1 (PDB ID code 1FJM). Aα was subsequently located using the atomic coordinates of free Aα (PDB ID code 1B3U). The solution was examined and modified using O (Jones et al., 1991) and refined using CNS (Brunger et al., 1998). The structures were refined to 2.6 and 2.8 Å resolution, respectively, for the core enzyme bound to OA and MCLR. The final refined atomic models contain residues 6–294 for Cα and 9–589 for Aα.

Methylation of PP2A Core Enzyme by LCMT

LCMT and PP2A core enzyme, at a 1:2 molar ratio, was incubated on ice. Methylation was initiated by addition of S-adenosyl methionine to a final concentration of 0.75 mM. The reaction was carried out at 22°C and reached completion after 2–3 hr. The methylated PP2A core enzyme was purified away from LCMT by anion-exchange chromatography.

Gel Filtration Chromatography

For each assay, 0.5 mg of the PP2A core enzyme was incubated with 0.5 mg of the regulatory subunit at 4°C for 10 min before the mixture was applied to Superdex 200 (10/30, GE Healthcare) in buffer containing 25 mM Tris (pH 8.0), 150 mM NaCl, and 2 mM DTT. The peak fractions were applied to SDS-PAGE and visualized following Coomassie blue staining.

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Accession Numbers

The atomic coordinates of the PP2A core enzyme bound to OA and MCLR have been deposited in the Protein Data Bank with the ID codes 2IE4 and 2IE3, respectively.